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Archaea are the predominant and responsive ammonia oxidizing prokaryotes in a red paddy soil receiving green manures

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Abstract

Application of green manures is an effective approach to optimizing N management in paddy soils. Nitrification is a key process in the N cycle and ammonia oxidization is the first and typically limiting step in nitrification. In this study, we investigated the changes of ammonium oxidizing prokaryotes after the application of green manure in a red paddy soil using pot experiments. The experiment included four treatments; milk vetch-rice, radish-rice, ryegrass-rice and winter fallow-rice. The nitrification potential was measured, and the abundance and community of *amoA* genes from ammonia-oxidizing archaea (AOA) and bacteria (AOB) were quantified. The results showed that the AOA to AOB ratios ranged from 7 to 80, and that the milk vetch treatment increased the abundances of AOA and AOB. The abundance of AOA showed negative correlations with nitrification potential and NH_4^+-N, and positive correlation with soil pH in the acidic red paddy soil. DNA sequence analyses revealed that the *Nitrososphaera* and *Nitrosospira* were the dominant clusters of AOA and AOB, respectively. The dominant clusters of AOA were significantly changed by utilization of green manures, especially radish. Partial least squares path modeling analysis showed that green manures exerted larger effects on the abundances of AOA than on AOB, and the community structure of AOA had the strongest effect on nitrification potential. The high abundance of AOA found in this study and their responsiveness to green manuring suggests that AOA are critically important for soil ammonia oxidation in these soils and more sensitive to green manuring than AOB.

Key words: green manure; ammonia-oxidizing archaea; nitrification potential; driving factors; red paddy soil

1. Introduction

Nitrification is the conversion of inorganic nitrogen from a reduced form to an oxidized state [1]. Ammonia oxidization is the first and typically the rate-limiting step, carried out by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). The *amoA* gene, which can occur in both AOA and AOB is a good indicator of the abundance and diversity of ammonia oxidizing prokaryotes, and is commonly used as molecular biomarker in the studies of nitrification [2]. The abundance and communities of AOA and AOB are influenced by soil properties in agricultural soil. Many reports have revealed that pH is the main factor driving the community changes of AOA and AOB [2-6]. Substrate availability such as the amount of ammonia is also an important driver of both AOA and AOB species richness [2, 7, 8]. Other field management practices and soil properties such as fertility regime [9, 10], manure input [11, 12], soil moisture and temperature [13] may also affect the nitrification process and the distribution and activity of ammonia oxidizers. The relative contribution of AOA and AOB in different types of soils are still under debate [10]. Numerous studies have confirmed that AOA are predominate ammonia-oxidizing prokaryotes when compared to their bacterial counterparts in multiple terrestrial environments [14], such as those with alternating oxygenated/hypoxic conditions [15], and acidic soils [16], suggesting that AOA could have a potentially greater role in the overall nitrification than their bacterial counterparts [17].

The utilization of green manures in crop rotations is a management practice designed to improve soil fertility and reduce chemical fertilizer applications [18-20]. In southern China, the planting of winter green manures is used as an effective means of improving the productivity and sustainability of paddy fields under double-rice cropping systems [21, 22], and milk vetch (*Leguminosae*), radish (*Cruciferae*) and ryegrass (*Gramineae*) are popular green manure species in this area. The production practices have proved that all of these three kinds of green manure improved the grain yield and soil fertility, and the effects of these three green manures have differences, but the mechanisms are unclear. The characteristics of the plant residues differ between plant families, and the release of nutrients during decomposition may have various influences on the abundance and diversity of AOA and AOB. Many studies have investigated the ammonia oxidizers in different kinds of paddy soils with various management practices [4, 9, 23, 24]. However, the effects of different green manures on the abundance and community structure of ammonia oxidizers is not well understood. It is possible for us to find out the driving factors in nitrification by evaluating the responses of ammonia oxidizer on different green manures. After incorporation of green manures, the decomposition of plant residues and the priming effects may have more profound influences on soil conditions than the stage after rice harvest. In the period after rice cultivation, the interactive effects of green manure decomposition and rice growth may also change the nitrification process and activity of ammonia oxidizers. So, it is reasonable to compare the variations before and after rice cultivation.

In this study, we investigated the abundance and community diversity of ammonia oxidizers at the stages before rice transplantation and after rice cultivation in the winter green manure – double rice system. We hypothesized that (i) AOA *amoA* gene would be more abundant in this

89 acidic paddy soil thus indicate the predominant of AOA compared with AOB, (ii) the different
90 green manures had various effects on AOA and AOB, (iii) sampling stage may be one of the
91 driving factors that led to the changes of ammonia oxidizers.
92
93 **2. Materials and methods**
94 *2.1 Plant materials and soils*
95 Pot experiments were conducted in the Red Soil Experimental Station (26°45' N, 111°52' E;
96 elev. 150 m) of Chinese Academy of Agricultural Sciences, Qiyang, Hunan Province, China. The
97 paddy soil is derived from Quaternary red clay, and classified as a Ferralic Cambisol [25]. On
98 October 20, 2013, milk vetch (*Astraglus sinicus* L.), radish (*Raphanus sativus* L.) and ryegrass
99 (*Lolium multiflorum*) were planted in three field plots, with a winter fallow plot as a control
100 (weeds in the winter fallow plot were removed by hand and the plot was kept plant free). Soils and
101 plants were collected at the full-bloom stage of milk vetch and radish (March 25, 2014). The
102 aboveground parts of milk vetch, radish, ryegrass and their corresponding soils, as well as the soil
103 from the winter fallow plots were sampled for the use of following pot experiments. Soils was
104 collected to a depth of 0.2 m and sieved through a 2 mm mesh prior to use in the pot experiment.
105 The properties of green manures and soils are shown in Tables S1 and S2.
106
107 *2.2 Experimental design and sampling*
108 Four treatments were designed including milk vetch-rice (the milk vetch soil was
109 incorporated with the corresponding milk vetch, MV), radish-rice (the radish soil was incorporated
110 with the corresponding radish, RD), ryegrass-rice (the ryegrass soil was incorporated with the

111 corresponding milk ryegrass, RG) and winter fallow-rice (the milk vetch soil without plant
112 residues was incorporated, WF). Each treatment was conducted in triplicate with a completely
113 randomized design. The size of pots used in the experiments was 275 mm in height and 270 mm
114 in diameter, and a total of 10 kg soil was packed into each pot. The green manures were collected
115 at their full bloom stage, then the plants were chopped and weighed. The amount of green manure
116 incorporated in each pot was 200 g fresh biomass. Each pot was received 0.6 g N, 0.6 g P_2O_5 and
117 0.6 g K_2O (4 g 15-15-15 NPK compound fertilizer) as a basal fertilizer. All chemical fertilizer and
118 green manures were applied on March 25, 2014. Rice plants were transplanted 30 days after the
119 application of green manures. All pots were flooded after fertilization and during the growth of the
120 rice.
121 Soils were sampled at two stages: 1) 30 days after the incorporation of green manure (before
122 the rice was transplanted, on 25 April, Stage 1), and 2) after the rice was harvested (on 11 July,
123 Stage 2). After being mixed well and sieved (< 2 mm), soil samples were divided, one batch was
124 then stored at −20°C for DNA extraction, and a second batch was stored at 4°C prior to chemical
125 analyses. After the rice harvest, the grain, shoots and roots were sampled separately, dried and
126 milled to measure the yields and nutrient contents.
127
128 *2.3 Chemical analysis*
129 All soil analyses were conducted according to Lu [26]. Soil pH was measured using pH
130 electrode with a soil to water ratio of 1:2.5. Soil total nitrogen (TN) was determined using the
131 Kjeldahl method. Soil organic matter (SOM) and total C of the plants were determined with the
132 potassium dichromate oxidation method. Soil available phosphorus (AP) and available potassium

(AK) were extracted by 0.5 mol L^{-1} $NaHCO_3$ and 1 mol L^{-1} CH_3COONH_4 respectively. Soil NH_4^+-N and NO_3^--N were extracted by 2 M KCl, and measured on a continuous flow analyzer (AA3, SEAL, Germany).

2.4 Nitrification potential

Nitrification potential (NP) was measured using the chlorate slurry inhibition assay with slight modifications [1]. Briefly, each soil sample (5.0 g) was transferred to a 25 ml centrifuge tube including the 20 ml liquid medium (the concentration of NH_4^+-N was 100 mg L^{-1} and the pH of the medium was adjusted to 7.5 using H_2SO_4 or NaOH solution). The slurry with soil and liquid medium was incubated for 5h at 25°C, and 0.2 ml sodium chlorate (1 M) was added to inhibit the oxidization of nitrite to nitrate during the incubation. After incubation, 5 ml of 2 M KCl was added to the extract and the nitrite released during the incubation period was then determined on a continuous flow analyzer (AA3, SEAL, Germany).

2.5 DNA extraction and real time quantitative PCR

Three DNA extractions per sample were performed using a FastDNA Spin Kit for Soil (MP Bio, Santa Ana, CA, USA) following the manufacturer's procedures. Three extractions from each soil sample were pooled and DNA was then quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA). The DNA samples were stored at -80°C for further analysis.

Real-time quantitative PCR of archaeal and bacterial *amoA* genes were performed on 7500 Real time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, US) in triplicate. The 25 μL

155 reactions contained the following ingredients: 12.5μL of Power SybrGreen qPCR Master Mix
156 (Thermo Fisher Scientific Inc.), 0.5 μL each of 10 μM forward and reverse primers for
157 ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB), 9.5μL of ddH_2O and
158 2μL of 10-fold diluted extracted DNA. Cycling protocols were 5 min at 95°C followed by 40
159 cycles of 5s at 95°C, 30s at 56°C, and 40s at 72°C for both AOA and AOB *amoA* genes. The
160 primers for AOA were Arch-amoAF (STAATGGTCTGGCTTAGACG) and Arch-amoAR
161 (GCGGCCATCCATCTGTATGT) [27], with the amplicon length of 635 bp. The primers for
162 AOB were amoA-1F (GGGGTTTCTACTGGTGGT) and amoA-2R
163 (CCCCTCKGSAAAGCCTTCTTC) [28], with an amplicon length of 491 bp. To construct the
164 quantitative PCR standards, the purified PCR products were ligated into the pMD 18-T Vector
165 (Takara Biotech, Dalian, China), then transformed into the Top 10 chemically competent *E. coli*
166 (Takara Biotech). The extracted and purified vectors were used as standards by serial dilution of
167 the plasmids carrying the respective gene targets.
168
169 *2.6 DNA sequencing*
170 The PCR amplification of AOA and AOB *amoA* genes was undertaken on an ABI 9700
171 thermocycler (Thermo Fisher Scientific Inc.), using the same primers and cycling conditions as
172 described above for quantitative PCR. Barcodes and linkers were added to each forward primer.
173 The PCR products from different samples were quantified using the QuantiFluorTM-ST System
174 (Promega, Wisconsin, US) and pooled at equal concentrations. Amplicon sequencing was
175 performed on a pyrosequencing Roche GS FLX Titanium platform (454 Life Sciences, Branford,
176 CT, USA) according to the manufacture's protocols.

177

178 *2.7 Bioinformatics analyses*

179 DNA raw sequence data were subjected to systematic checks using the following criteria:
180 errors from PCR amplification and pyrosequencing were reduced using Usearch software (version
181 7.1) [29]. Briefly, reads quality was controlled using a sliding window approach: when the average
182 quality score within a 50-bp window dropped below 20, the sequence was trimmed, sequences
183 with mismatches to either the primer (> 2), with one or more ambiguous bases or with homologs
184 longer than 10 bp, or sequence lengths < 200 bp were removed. The remaining high-quality
185 sequences were screened using UCHIME [30] to discriminate and eliminate putative chimeras.
186 After quality control, a total of 241,820 and 168,398 high quality raw sequence reads were
187 obtained for AOA and AOB, respectively.

188 The valid sequences obtained were clustered into operational taxonomic units (OTUs) using
189 Usearch software (version 7.1) with a sequence identity threshold of 97% for both AOA and AOB
190 *amoA* gene. The duplicated sequences were removed using the fastx_unique command, and the
191 singletons were discarded using the sortbysize derep.fasta command in Usearch. A total of 64 and
192 51 OTUs were obtained for AOA and AOB, respectively. The representation sequences of each
193 OTU of the *amoA* gene were aligned against the Fungene database [31].

194 The diversity statistics and rarefaction were calculated subsequently. To eliminate the bias of
195 libraries' alpha diversity comparison, the same number of sequences in each pyrosequencing
196 library was subsampled randomly (6853 reads for AOA and 5230 reads for AOB) by Mothur [32]
197 (http://www.mothur.org/wiki/Schloss_SOP). Good's coverage [33] of AOA and AOB in all the 24
198 samples exceeded 99% (Table S1), revealing that these libraries could well reflect the diversity of

199 archaeal and bacterial *amoA* gene. Chao richness [34] and Shannon index [35] were calculated to
200 describe the community's richness and diversity using Mothur [32]. The results of Chao richness
201 and Shannon index of AOA and AOB *amoA* genes were listed in Table S3. phylogenetic trees
202 were constructed based on the OTUs with relative abundance > 0.1% of AOA and AOB *amoA*
203 gene, by the neighbor-joining method using a Kimur 2-parameter distance with 1,000 bootstrap
204 replicates with MEGA 6 [36].
205 All original nucleotide sequence reads were deposited at the NCBI Sequence Read Archive
206 (SRA) with the accession number of SRP091934.
207
208 *2.8 Statistical analyses*
209 One way analysis of variance (ANOVA) and correlation analysis was conducted using SAS
210 8.1, and the Duncan's test was employed to evaluate significance ($p < 0.05$) in ANOVA. The
211 relationship between green manure, soil properties, NP, abundances and communities of AOA and
212 AOB were explored using partial least squares path modeling (PLS-PM). PLS-PM is a statistical
213 method for studying complex multivariate relationships among observed and latent variables [37].
214 The data of AOA abundance and community at S1 stage and AOB at S1 stage were used to
215 construct the model. Five latent variables were used: Green manure, soil properties (Soil), NP,
216 abundance and communities of AOA and AOB. Carbon to nitrogen ratios of the green manures, N
217 input, P input and K input from the green manures were selected as the manifest variables (i.e.
218 observed variables) to reflect the latent variable green manure; pH, SOM, TN, NH_4^+-N, NO_3^--N
219 and AK were selected as the manifest variables of soil properties; *amoA* gene copy numbers of
220 AOA and AOB were the observed variables of AOA and AOB abundances, respectively; the

relative abundances of main OTUs of AOA and AOB (12 abundant OTUs for each library) were the observed variables to reflect the community structure of AOA and AOB, respectively. The goodness of fit (GoF) index was calculated as the geometric mean of the average communality and R^2 value in the model and assess the overall prediction performance of the model [37]. The R package "plspm" [38] was used to construct the model.

3 Results

3.1 Response of soil chemical properties to green manuring

Some soil properties changed significantly following the application of green manures (Table 1). At Stage 1, 30 days after incorporation, RD decreased the total N and NO_3^--N contents ($p < 0.05$). Available K increased significantly independently of which green manure was applied ($p < 0.05$). After the rice was harvested (Stage 2), green manure treatments had significantly lower pH values than WF ($p < 0.05$). Compared with WF, MV decreased NH_4^+-N but increased the NO_3^--N content; RD increased soil Total N and the available-P content; RG decreased NH_4^+-N and increased the available-P content ($p < 0.05$). At Stage 2, all the soil chemical properties except pH and available P were lower than those at Stage 1 (Table 1).

3.2 Response of the nitrification potential

Soil nitrification potential ranged from 11.75 to 51.54 ng N g^{-1} h^{-1}, and was higher at Stage 1 than that at Stage 2. At Stage 1, the incorporation of radish increased the soil nitrification potential compared with WF and MV. After harvesting, there were no significant differences between treatments (Fig 1).

243

244 *3.3 Quantification of archaeal and bacterial amoA genes and their correlations with soil*

245 *properties*

246 Archaeal *amoA* genes, as indicators for AOA, were always more abundant than those of

247 bacteria, indicating the presence of AOB. The ratios of AOA to AOB *amoA* genes ranged from 7

248 to 80. MV increased both the AOA and AOB *amoA* genes (Fig. 2). For the AOA *amoA* gene, MV

249 were 3.1 and 1.6 times higher than that in WF at Stage 1 and Stage 2, respectively. For the AOB

250 *amoA* gene, MV were 6.5 and 3.7 times higher than WF at Stage 1 and Stage 2, respectively. RG

251 also increased the AOA and AOB *amoA* gene at Stage 2 significantly, while RD had no effect on

252 the abundance of both AOA and AOB *amoA* genes (Fig. 2).

253 Statistical analysis of the relationships between AOA and AOB *amoA* genes abundances and

254 soil NP, pH, NH_4^+-N (Fig 3) demonstrated that abundances of AOA *amoA* gene correlated

255 negatively with NP and NH_4^+-N ($p < 0.05$), and positively with pH ($p < 0.01$). No significant

256 correlations were observed between the abundance of AOB *amoA* gene and NP, pH, and NH_4^+-N.

257

258 *3.4 Diversity of amoA genes from archaea and bacteria*

259 The most abundant OTUs (relative abundance higher than 0.1%) of *amoA* genes from

260 archaea and bacteria were used to construct the phylogenetic trees. The results showed that the

261 most abundant OTUs of AOA *amoA* were mainly affiliated with *Nitrososphaera* (group1.1 b) and

262 *Nitrosotalea* (group 1.1 a-associated) cluster (Fig 4A). The number of reads affiliated to the

263 *Nitrososphaera* and *Nitrosotalea* clusters accounted for 85.0% and 14.3% of total reads,

264 respectively. The archaeal *amoA* OTUs of *Nitrososphaera* were distributed among four subclusters.

265 Subcluster 1 included the most abundant sequences, accounting for 79.7% of the total sequences.
266 The affiliation of the *amoA* sequences detected in this study to the four different subclusters and
267 their abundances are shown in Fig 4B. RD was associated with clear changes in the composition
268 of AOA *amoA* which significantly increased the relative abundance of the *Nitrosotalea* cluster
269 (group 1.1a-associated), subcluster 2 and subcluster 3 of *Nitrososphaera*, and decreases in
270 Subcluster 1 at both sampling stages compared with WF ($p < 0.05$). The MV and RG treatments
271 influenced the composition of AOA *amoA* by changing the relative abundance of some subclusters.
272 MV significantly decreased the relative abundance of subcluster 2 of *Nitrososphaera* at Stage 1
273 and increased the subcluster 3 of *Nitrososphaera* at Stage 2 ($p < 0.05$); RG significantly increased
274 the subcluster 4 of *Nitrososphaera* at Stage 2 ($p < 0.05$).
275 The main OTUs of AOB *amoA* were grouped into *Nitrosospira* and *Nitrosomonas* clusters,
276 accounting for 99.3% and 0.2% of all the sequences, respectively (Fig. 5A). Five subclusters of
277 *Nitrosospira* were found. They were Cluster 3a (0.3%), Cluster 3b (2.6%), Cluster 9 (2.7%) and
278 Cluster 12 (91.0%), and another 2.6% of the sequences were clustered into *Nitrosospira* but didn't
279 belong to any of the known subclusters. Cluster 12 were the dominant cluster in all the treatments
280 at both sampling stages. This cluster was not affected by the application of green manures (Fig
281 5B), while the abundance of other subclusters had minor variation after green manuring compared
282 with WF. MV had a lower relative abundance of the unclassified cluster of *Nitrosospira* at Stage 2;
283 Radish had higher relative abundance in cluster 3a at Stage 1; Ryegrass had higher relative
284 abundance in cluster 3b and lower relative abundance in the unclassified cluster of *Nitrosospira* at
285 Stage 2 ($p < 0.05$) (Fig. 5B).
286

3.5 Partial least squares path modeling analysis

The PLS-PM model evaluated the correlations between green manure treatments, soil properties, NP, abundance and the communities of AOA (Fig 6A) and AOB (Fig 6B) at the first sampling stage. In the AOA model, green manure application had positive and soil properties had negative effects on the abundance of AOA, the path coefficients were 1.237 and -1.239, respectively. Nitrification potential was affected by the abundances and communities of ammonia oxidizers. In this model, the community of AOA had positive effects while their abundance had small negative effects on NP, indicating that the community of AOA might be the main factor that affect nitrification potential. In the model of AOB, soil properties had positive effects on the AOB community (the path coefficient was 0.842), both the abundance and community of AOB had no significant effects on nitrification potential.

The bi-group analysis was conducted between the two models to compare the differences of the structural coefficients, using the bootstrap t-test method with 1000 resamples to calculate the standard error estimates. The results of bi-group analysis showed that none of the path coefficients between AOA and AOB were significantly different, indicating that the green manure and soil properties had similar effects on AOA and AOB. The GoF index is a pseudo Goodness of fit measure to show the model quality at both the measurement and structural models [37]. The GoF of the two model in our study were 0.53 for AOA and 0.51 for AOB, respectively, suggesting that the overall prediction performance of the models were acceptable.

4 Discussion

AOA rather than AOB *amoA* gene were found to predominant in abundance at both sampling

309 stages in this study, i.e. 30 days after incorporation of green manure and after the rice was
310 harvested. The soil we studied is a typical of acidic paddy soils in southern China. Many studies
311 reported that AOA had a competitive advantage over AOB in acidic soils [3, 5, 39], and that AOA
312 were better adapted to the alternation between oxygenated and hypoxic conditions [15, 40]. Some
313 studies showed that the abundance and community of AOB were changed by inorganic nitrogen
314 fertilizer such as ammonium based fertilizers or urea [11, 41]. By contrast, AOA abundance and
315 community structure has been shown to change due to the application of the organic substrates
316 and the combined application of organic and chemical fertilizers [11, 42]. In our study, AOA *amoA*
317 was more sensitive to the application of green manures than AOB *amoA*, which is consistent with
318 former studies [14, 41]. The utilization of green manure in paddy soils is an effective and valuable
319 management approach to maintaining the sustainable development of agriculture. The
320 decomposition of green manures following their incorporation releases large quantities of nitrogen
321 and carbon [43]. The mineralized N released by green manures could be considered similar to that
322 provided by organic fertilizer, and might favor AOA and promote their growth and activity. The
323 greater abundance and sensitivity of AOA *amoA* to the application of green manures indicated that
324 AOA might play more important roles in winter green manure-rice cropping system than AOB.
325 Nitrification potential may depend not only on population size but also on community
326 composition. The change of communities structures of ammonia oxidizers may also lead to
327 different responses in nitrification potential [12]. Our results suggest that AOA rather than AOB
328 controlled nitrification in this system. The predominance of AOA in red paddy soils may be partly
329 because of the acidic soil environment in which different green manures led to the differential
330 responses of ammonia oxidizers and nitrification potential.

Paddy soil provides a complex environment for ammonia oxidizers. Within this environment, soil conditions such as temperature [44], pH [4, 5], nutrient content [12] and their interactions have various influences on AOA and AOB. The growth of green manures and decomposition of plant residues after incorporation adds significant quantities of organic C and N into soil. The nitrogen mineralized from incorporated plants is one of the substrates in nitrification and might stimulate the growth of ammonia oxidizing prokaryotes. The C/N of milk vetch, radish and ryegrass used in this study were 11.72, 14.89 and 16.83, respectively, and litter quality of these three green manures were different. Plant litter quality is an important factor regulating decomposition and nutrient cycling [45]. The different mineralization characteristics of these three green manures resulted in various effects on ammonia oxidizers. Application of milk vetch increased the abundance of AOA and AOB *amoA* genes, while radish increased the nitrification potential and changed the distribution of dominant groups in AOA *amoA*. These results suggest that green manures had different effects on nitrification and ammonia oxidizing prokaryotes, and the difference may because of the different characteristics of the green manure crops.

The dominant groups in our study were *Nitrososphaera* of AOA and *Nitrosospira* of AOB, which are commonly detected in many soil environments [4, 17, 46]. Previous studies have recognized the *Nitrososphaera* cluster as the most abundant soil AOA lineage in both acidic and alkaline soils [42]. The *Nitrososphaera* cluster was detected with a strong genetic capacity to utilize various ammonia sources and was directly linked with nitrification activity in agricultural soils [16, 47, 48]. The *Nitrosotalea* cluster was also detected in our study. It was defined as an obligate acidophilic AOA, and provides a capacity for nitrification in acidic soils [49]. The existence of *Nitrosotalea* in our acidic red paddy soil was confirmed with these studies. For AOB,

353 *Nitrosospira* were dominant in relatively low N environments, while *Nitrosomonas* were prevalent
354 in environments with high N loadings [46]. Our study observed that most sequences of AOB were
355 affiliated with the *Nitrosospira* cluster 12. These findings are consistent with previous studies that
356 identify that AOB are dominated by *Nitrosospira* cluster 12 in acid red paddy soils, and the AOB
357 distribution was not affected by different fertilization treatments [23].

358 In conclusion this study suggested that AOA and AOB have a quantitatively different
359 importance in red paddy soil and that mainly AOA respond to green manuring. These results
360 support emerging evidence of different ecological niche preferences of AOA and AOB in soils.

361

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367

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Table 1. Soil chemical properties in different treatments at different growth stages

Treatment	pH	SOM ($g\ kg^{-1}$)	TN ($g\ kg^{-1}$)	NH_4^+-N ($mg\ kg^{-1}$)	NO_3^--N ($mg\ kg^{-1}$)	Available P ($mg\ kg^{-1}$)	Available K ($mg\ kg^{-1}$)
30 days after green manure incorporated (S1)							
MV	5.12 a	40.9 a	1.83 a	31.2 a	88 a	94 a	169 b
RD	5.12 a	41.6 a	1.77 b	41.0 a	77 a	97 a	194 a
RG	5.18 a	39.6 a	1.81 a	28.0 a	55 b	98 a	208 a
WF	5.04 a	34.0 a	1.85 a	29.3 a	84 a	96 a	131 c
After rice harvesting (S2)							
MV	5.56 b	34.2 a	1.70 b	11.6 b	26 a	123 a	28 b
RD	5.52 b	31.0 a	1.77 a	18.4 ab	15 ab	124 a	29 b
RG	5.57 b	29.0 a	1.72 ab	12.4 b	18 ab	120 ab	40 a
WF	5.76 a	29.0 a	1.69 b	23.0 a	8 b	111 b	26 b

MV: milk vetch-rice; RD: radish-rice; RG: ryegrass-rice; WF: winter fallow-rice. Values are means ±SE (n=3).

Means followed by different letters are significantly different ($p < 0.05$).

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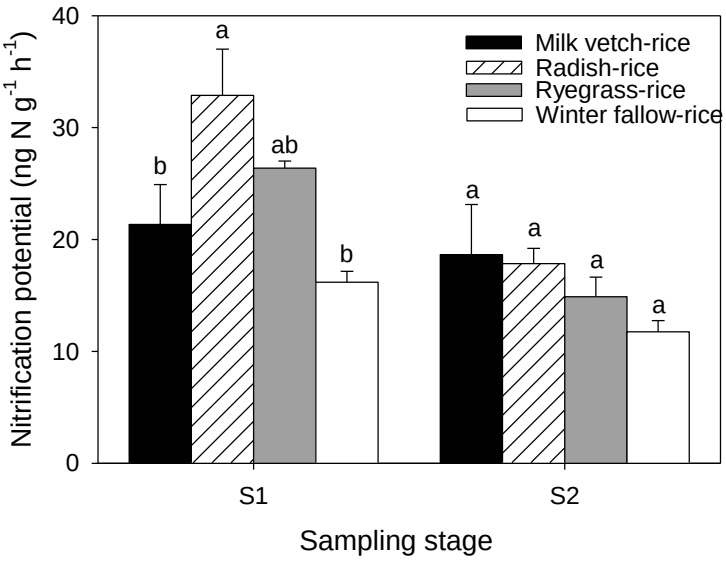

506

507 **Fig 1. Soil nitrification potential in different treatments at the two sampling stages. Vertical T bars indicate**

508 **SE. Bars topped by different letters are significantly different ($p < 0.05$).**

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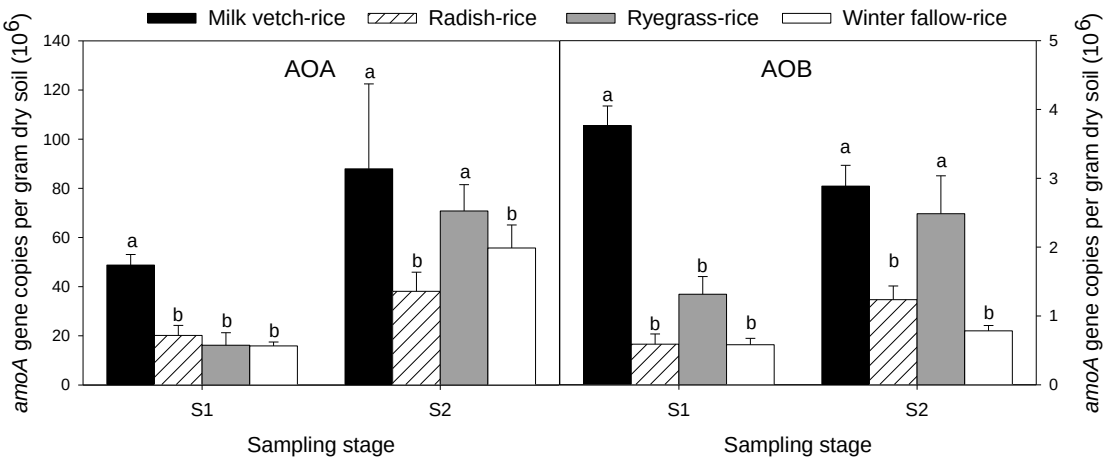

510

511 **Fig 2. Ammonia-oxidizing archaeal *amoA* abundance (AOA) and Ammonia-oxidizing bacteria *amoA***

512 **abundance (AOB) in different treatments at two sampling stages. Vertical T bars indicate SE. Bars topped**

513 **by different letters are significantly different ($p < 0.05$).**

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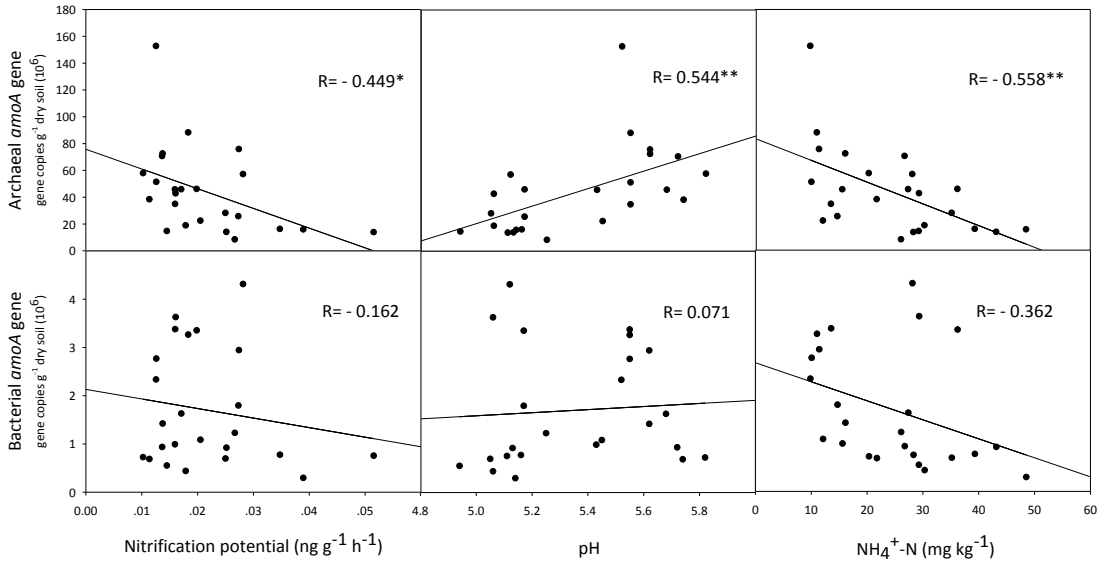

515

516 **Fig 3. Relationships between archaeal and bacterial *amoA* gene abundances and soil NP, pH, NH_4^+-N. * $p <$**

517 **0.05, ** $p < 0.01$, n=24.**

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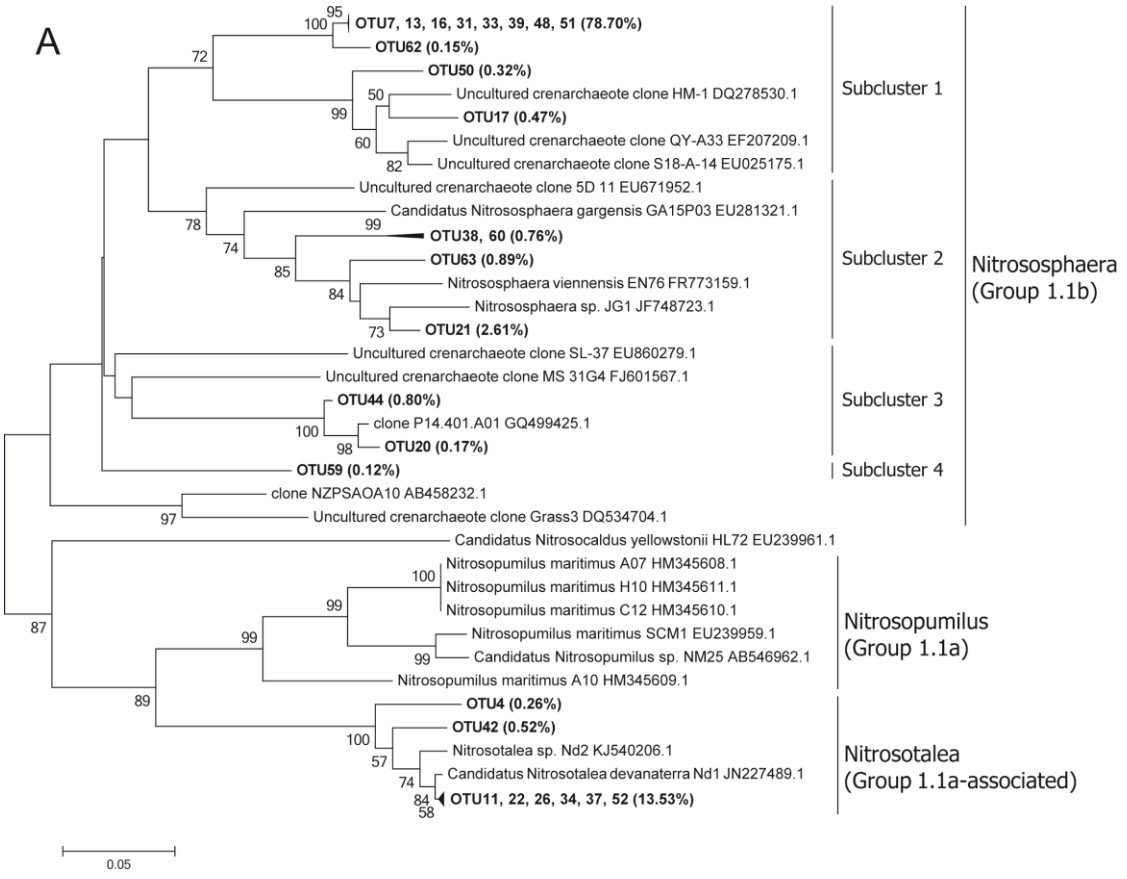

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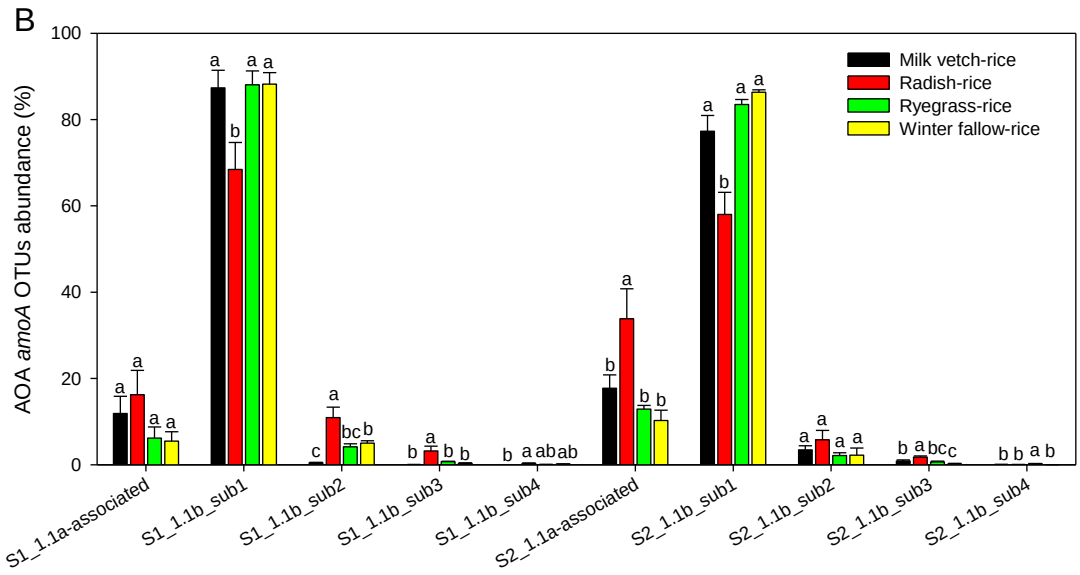


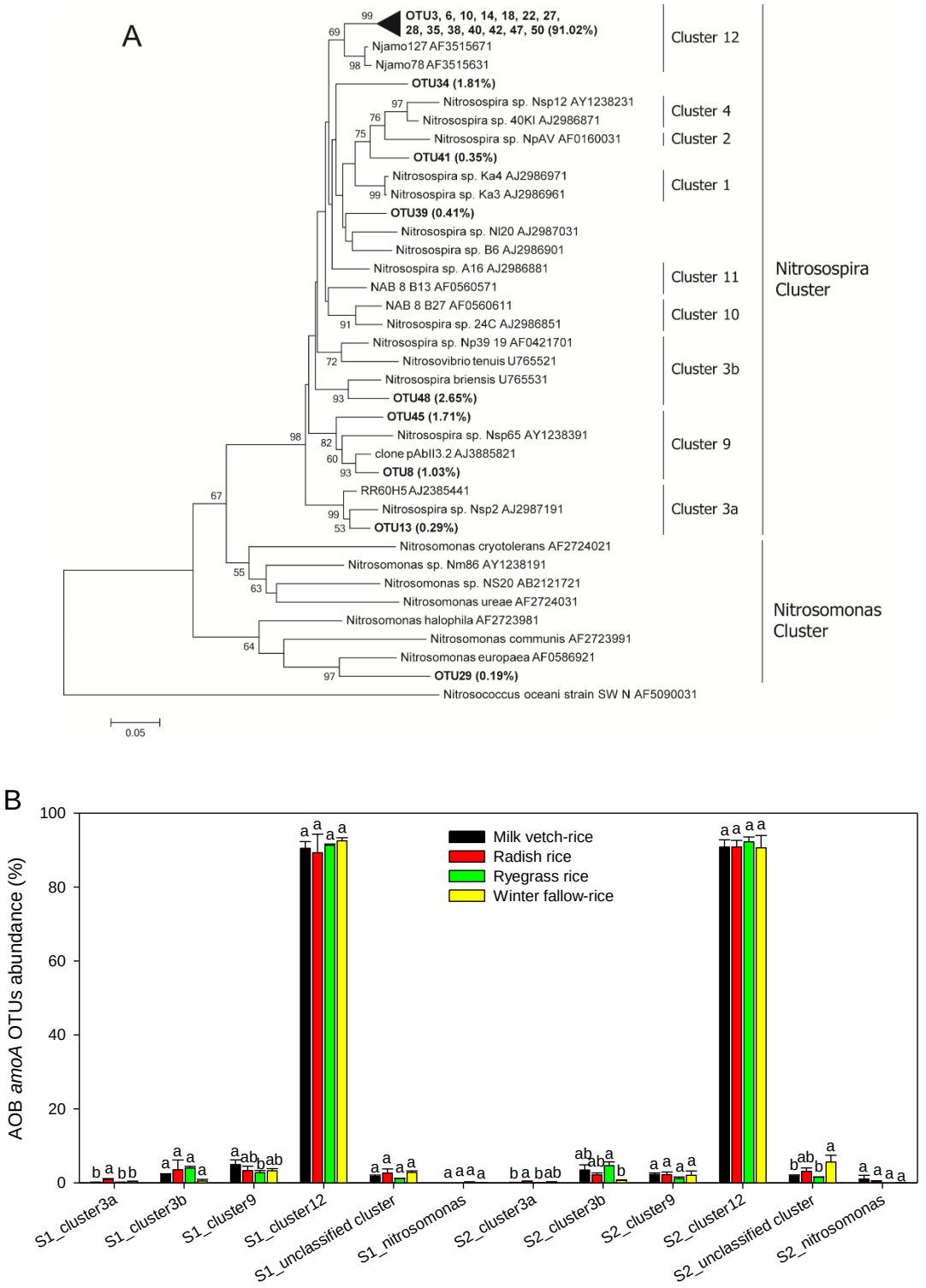
520

521 **Fig 4. A) A Neighbor joining tree for AOA partial *amoA* OTUs (representatives with relative abundance >**

522 **0.1%). AOA *amoA* OTUs in this study are shown in bold. The scale bar represents 5% nucleic acid sequence**

523 **divergence, and bootstrap values of > 50% are showed at branch points. B) The composition analysis of**

524 **AOA *amoA* gene in the four treatments at the two sampling stages.**



525

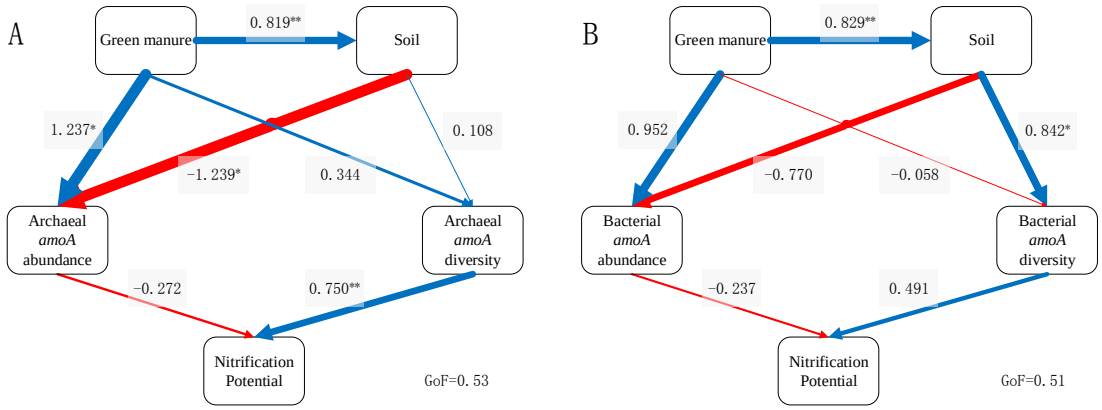
526

527 **Fig 5. A) A neighbor joining tree for AOB partial *amoA* OTUs (representatives with relative abundance >**

528 **0.1%). AOB *amoA* OTUs in this study are shown in bold. The scale bar represents 5% nucleic acid sequence**

529 **divergence, and bootstrap values of > 50% are showed at branch points. B) The composition analysis of**

530 **AOB *amoA* gene in the four treatments at the two sampling stages.**



531

532 **Fig 6. Directed representation of the Partial Least Squares Path Model (PLS-PM). AOA at Stage 1 (A) and**

533 **AOB at Stage 1 (B) were analyzed separately. Only latent variables were represented in the graph. Green**

534 **manures represented the different treatments. Soil represented soil properties. Archaeal *amoA* diversity and**

535 **Bacterial *amoA* diversity represented the distribution of AOA *amoA* OTUs and AOB *amoA* OTUs. Indicated**

536 **value are the path coefficients. Larger path coefficients were reflected in the width of the arrow; blue arrow**

537 **indicated a positive effect, while red arrow indicated a negative effect. Path coefficients that differ**

538 **significantly from 0 were indicated by $^{*}p < 0.05$ and $^{**}p < 0.01$. Significance was based on 1000 resampled**

539 **bootstraps. GoF indicated the Goodness of Fit, a measure of the overall prediction.**

540

541